AD_____

Award Number: DAMD17-03-1-0459

TITLE: Characterization of Novel Genes within 8P11-12 Amplicon in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Zeng-Quan Yang

CONTRACTING ORGANIZATION: The University of Michigan
Ann Arbor, MI 48109-1274

REPORT DATE: June 2005

TYPE OF REPORT: Annual Summary

20060309 144

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**
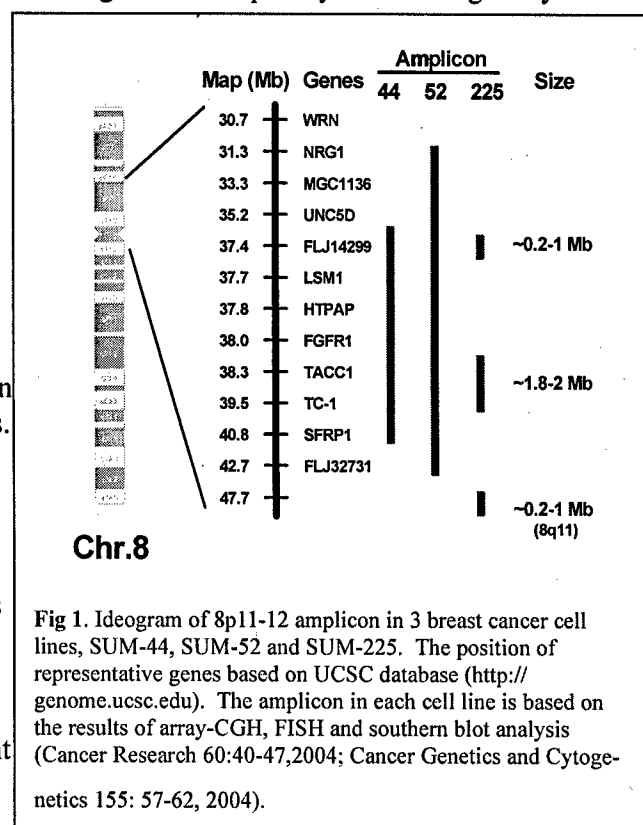
1. REPORT DATE (DD-MM-YYYY) 01-06-2005	2. REPORT TYPE Annual Summary	3. DATES COVERED (From - To) 1 Jun 2004 – 31 May 2005

4. TITLE AND SUBTITLE
Characterization of Novel Genes within 8P11-12 Amplicon in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0459

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Zeng-Quan Yang

E-mail: yangz@karmanos.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Michigan
Ann Arbor, MI 48109-1274

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The development of breast cancer is associated with gene amplification and overexpression that are believed to have a causative role in oncogenesis. An important challenge in breast cancer research is to identify and characterize these genetic changes. Focal amplifications involving chromosome 8p11-p12 occur in approximately 15~20% of primary, uncultured human breast cancers. Recently, we have undertaken a detailed genomic and expression analysis of the 8p11-p12 amplicon in breast cancer cell lines and identified several novel candidate genes including TC-1 and FLJ14299. We observed that TC-1 is located at the common core-amplified domain of the 8p11-12 region and overexpressed in the subset of breast cancer cells. Furthermore, we have found that TC-1 has properties of an oncogene: TC-1 expression in normal mammary epithelial cell line MCF10A increases growth rate and allows growth in soft agar. Notably, suppression of TC-1 expression by siRNA inhibited cell proliferation in TC-1 over expressing breast cancer cell lines. Our recent date also suggested that cooperative activity of oncogenes including TC-1 within amplicons is crucial for inducing the specific transforming phenotypes in breast cancer.

15. SUBJECT TERMS
Amplicon, oncogen

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	14	19b. TELEPHONE NUMBER (include area code)

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction:

The development of breast cancer is associated with gene amplification and overexpression that are believed to have a causative role in oncogenesis(1-4). An important challenge in breast cancer research is to identify and characterize these genetic alternations. Global genomic and transcriptomic analysis have led to important insights in our understanding of the complexity and heterogeneity of this disease, and providing new avenues for the discovery of genetic alternation in human breast cancer (HBC). Recently we have performed the cytogenetic and molecular genetic profile of the SUM panel breast cancer cell lines. Of specific relevance to this application, we have found that three cell lines have overlapping amplicons in the short arm of chromosome 8 (Fig. 1). From this amplicon, we identified several novel candidate genes including TC-1 (C8ORF4), FLJ14299 and others(5). TC-1 is a novel gene highly expressed in thyroid cancer and some fraction of breast cancers. FLJ14299 contains a C2H2-like motif, which is also present in several tumor-related genes. Aberrant expression of TC-1 and FLJ14299 could be related to development and progression in breast cancer. The specific aims as outlined in this proposal will help us to better understand the biological function and genetic pathway of new target genes of this amplicon and identify better prognostic and predictive markers for an important subset of breast cancer.

Fig 1. Ideogram of 8p11-12 amplicon in 3 breast cancer cell lines, SUM-44, SUM-52 and SUM-225. The position of representative genes based on UCSC database (http://genome.ucsc.edu). The amplicon in each cell line is based on the results of array-CGH, FISH and southern blot analysis (Cancer Research 60:40-47,2004; Cancer Genetics and Cytogenetics 155: 57-62, 2004).

Body:

Task 1. To test the mechanistic significance of two novel genes, *FLJ14299* and *TC-1,* found to be amplified and over expressed in the SUM-44, SUM-52 and SUM-225 breast cancer cell lines (Months 1-20)

In our first annual report, we demonstrated that TC-1 has oncogenic properties. Overexpression of TC-1 in a spontaneously immortalized normal mammary epithelial cell line, MCF10A, enhanced cell proliferation in tissue culture. More important, TC-1 overexpression significantly promoted colony formation in soft agar assay relative to control vector alone. These results support the notion that TC-1 can facilitate cell growth and anchorage-independent growth associated with transformed phenotypes. Overexpression of FLJ14299 in MCF10A cells did not enhance cell proliferation and anchorage-

independent growth (Fig. 2). In addition, suppression of TC-1 expression by siRNA inhibited cell proliferation in TC-1 over expressing breast cancer cell lines.

In order to investigate the individual and cooperating oncogenic properties of newly identified genes in 8p11-12 amplicon, we established a cDNA expression library containing 8 candidate genes including TC-1 and FLJ14299 using a lentiviral expression system in this year. We transduced cells with several different combinations of genes from the lentiviral library into MCF10A cells and selected recipient cells in serum-free medium lacking EGF or IGF (Fig.3). RT-PCR with genes and vector-specific primers were used to detect transduced genes in cell clones that acquired EGF independence. Our primary data revealed that a combination of TC-1 and one of other two genes was sufficient to induce EGF independent growth (Manuscript in preparation). Clones containing only one of the three aforementioned genes were unable to survive in EGF-free medium. This result suggested that cooperative activity of oncogenes including TC-1 within amplicons is crucial for inducing the specific transforming phenotypes in breast cancer.
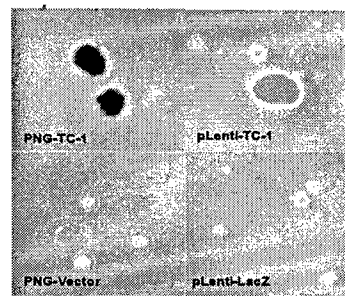

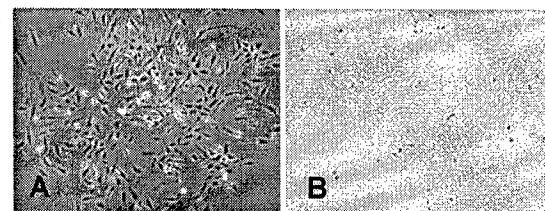**Fig. 2.** TC-1 expression enhances anchorage-independent growth in vitro

Fig. 3. (A) Emergence of EGF-independence colonies in 8p11-12 genes lentiviral library infected HME cells. (B) No cell growth in Lentiviral-LacZ infected cells.

Task 2. To detect potential downstream target genes of *TC-1* and *FLJ14299* overexpression using human cancer array and to investigate their particular tumor-related biological phenomena (Months 8-36)

TC-1 is a novel gene that was originally cloned from suppression subtractive hybridization between papillary thyroid carcinoma and its surrounding normal thyroid tissue(6). Recently, the structural characterization of the TC-1 protein revealed it is a natively disordered protein(7). Disordered proteins have been suggested to play roles in cell-cycle control, signal transduction, transcriptional and translational regulation. Using high-density oligonucleotide HG-U133A array, we identified a large number of differentially expressed genes in response to the enforced expression of TC-1. With a twofold change cutoff, we identified 79 probe sets with increased expression levels and 69 probe sets with decreased expression levels for MCF10A-TC-1 cells compared with MCF10A-PNG cells. Focusing on genes showing increased levels of expression, the dramatically overexpressed gene is TC-1 itself. Several up-regulated genes by TC-1 including matrix Gla protein (MGP), Wiskott-Aldrich syndrome protein interacting protein (WASPIP) and cadherin 11 that have been reported in various types of tumor including breast cancer with overexpression. From our initial analysis, it is impossible to determine whether the identified differentially regulated genes were true TC-1 targets or if they were regulated as a consequence of TC-1 activity. Therefore, validation of microarray results using independent techniques is necessary in future.

Task 3. To determine the amplification and overexpression pattern of 8p11-12 genes in primary breast cancer and to determine their associations with tumor phenotype and prognosis (Months 6-36)

To determine the amplification pattern of 8p11-12 genes in primary breast cancer, quantitative PCR analysis was carried out using genomic DNA obtained from 90 breast cancer specimens. The PCR experiments were performed using primers specific for *FLJ14299, LSM1, FGFR1* and *TC-1,* as these genes span the 8p11-12 amplicon detected in the cell lines(5). Of the 90 breast cancers examined, 25 showed evidence of high level amplification (greater than 4-fold) in at least part of the 8p11-12 region. Interestingly, *TC-1* and *FLJ14299* were most commonly amplified, while *FGFR1* was only found to be greater than 4-fold amplified in 4 of 90 primary breast cancers (Table 1). These results suggest that genes such as TC-1 and FLJ14299 flanking the *FGFR1* locus may be of greater significance in breast cancers. The analysis of expression pattern of these genes and their associations with clinical parameters are ongoing.

Table 1: TC-1 amplification in primary breast cancer specimens

FLJ14299	LSM1	FGFR1	TC-1
23/90	4/90	4/90	8/90

Training accomplishments:

With the help of this training award, my ability in critical thinking and problem solving in research work, the skills needed to formulate and carry out research, and to report findings in peer-reviewed journals have been dramatically improved. From October 1, 2004, I became an Research Assistant Professor in the Department of Pathology, Wayne State University School of medicine, and a member of the Breast cancer research program of the Karmanos Cancer Center.

Key Research Accomplishments:

1, Novel gene TC-1 of 8p11-12 amplicon has oncogenic properties in breast cancer.

2, Cooperative activity of oncogenes including TC-1 within 8p11-12 amplicons is crucial for inducing the specific transforming phenotypes in breast cancer.

3, Quantitative PCR analysis revealed that genes such as TC-1 and FLJ14299 that flanking the FGFR1 locus may be of greater significance of breast cancer.

Reportable Outcomes:

Manuscripts:
> Yang Z-Q, Albertson D and Ethier S. Genomic organization of the 8p11-12 amplicon in three breast cancer cell lines. Cancer Genetics and Cytogenetic. 2004; 155:57-62

Abstracts:
1. Yang Z-Q, Ray M, Grewal NAlbertson D and Ethier S. Identification and characterization of TC-1: a novel oncogene in human breast cancer. 2005 American Association for Cancer Research Annual Meeting in Anaheim, California. April 16-20, 2005.
2. Yang Z-Q and Ethier S. Characterization of the 8p11-12 amplicon in breast cancer. Era of Hope, Department of Defense Breast Cancer Research Program Meeting in Philadelphia, Pennsylvania, June 8-12, 2005

Conclusions:

We have made significant progress in the past year in characterizing two novel genes, TC-1 and FLJ14299, within 8p11-12 amplicon of breast cancer. Overexpression of TC-1 in normal human mammary epithelial cells promotes cell growth and anchorage-independent growth. In addition, we demonstrated that cooperative activity of oncogenes including TC-1 within amplicons is crucial for inducing the specific transforming phenotypes in breast cancer. Detailed analysis of amplification pattern of 8p11-12 genes in primary breast cancer demonstrated that TC-1 and FLJ14299 are most commonly amplified.

References:

1. Deming, S. L., Nass, S. J., Dickson, R. B., and Trock, B. J. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br J Cancer, *83:* 1688-1695, 2000.
2. Hui, R., Campbell, D. H., Lee, C. S., McCaul, K., Horsfall, D. J., Musgrove, E. A., Daly, R. J., Seshadri, R., and Sutherland, R. L. EMS1 amplification can occur independently of CCND1 or INT-2 amplification at 11q13 and may identify different phenotypes in primary breast cancer. Oncogene, *15:* 1617-1623, 1997.
3. Borg, A., Baldetorp, B., Ferno, M., Olsson, H., and Sigurdsson, H. c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. Int J Cancer, *51:* 687-691, 1992.
4. Kreipe, H., Feist, H., Fischer, L., Felgner, J., Heidorn, K., Mettler, L., and Parwaresch, R. Amplification of c-myc but not of c-erbB-2 is associated with high proliferative capacity in breast cancer. Cancer Res, *53:* 1956-1961, 1993.
5. Ray, M. E., Yang, Z. Q., Albertson, D., Kleer, C. G., Washburn, J. G., Macoska, J. A., and Ethier, S. P. Genomic and expression analysis of the 8p11-12 amplicon in human breast cancer cell lines. Cancer Res, *64:* 40-47, 2004.
6. Chua, E. L., Young, L., Wu, W. M., Turtle, J. R., and Dong, Q. Cloning of TC-1 (C8orf4), a novel gene found to be overexpressed in thyroid cancer. Genomics, *69:* 342-347, 2000.
7. Sunde, M., McGrath, K. C., Young, L., Matthews, J. M., Chua, E. L., Mackay, J. P., and Death, A. K. TC-1 is a novel tumorigenic and natively disordered protein associated with thyroid cancer. Cancer Res, *64:* 2766-2773, 2004.

APPENDICES:

<u>Yang Z-Q</u>, Albertson D and Ethier S. Genomic organization of the 8p11-12 amplicon in three breast cancer cell lines. Cancer Genetics and Cytogenetics 2004; 155:57-62

Genomic organization of the 8p11~p12 amplicon in three breast cancer cell lines

Zeng-Quan Yang[a], Donna Albertson[b], Stephen P. Ethier[a],*

[a]*Department of Radiation Oncology, University of Michigan Medical School, 7312 CCGC, PO Box 0948, 1500 East Medical Center Drive, Ann Arbor, MI 48109–0948*
[b]*Cancer Research Institute, University of California San Francisco, San Francisco, CA*

Received 16 December 2003; received in revised form 16 March 2004; accepted 19 March 2004

Abstract

Amplification of chromosomal regions leads to an increase of DNA copy number and expression of oncogenes in human breast cancer (HBC). Amplification of the 8p11~p12 region occurs in 10–15% of primary, uncultured HBCs. In our panel of 11 breast cancer cells, three cell lines, SUM-44, SUM-52, and SUM-225, have overlapping amplicons in the 8p11~p12 region. To characterize genome structure of the amplified regions, we performed fluorescence in situ hybridization using 8p11~p12 BAC clones in the 3 cell lines. The results revealed that the 8p11~p12 amplicon has a highly complex structure and that *FGFR1* is not in the common core-amplified domain in 3 breast cancer cell lines with the amplicon. These 3 cell lines provide good models for genetic and functional studies of candidate oncogenes of the 8p11~p12 region. © 2004 Elsevier Inc. All rights reserved.

1. Introduction

Genomic amplification is often observed in many types of human tumors, including human breast cancer (HBC). Oncogenes such as *ERBB2* (17q12), *CCND1* (11q13), and *C-MYC* (8q24), are activated by amplification and play a role in the development of some fraction of HBCs [1–4]. Recently detailed analyses of genomic structures and sequences of amplified regions such as those found at 11q13, 17q12~q23, and 20q12 have revealed that amplicons have complex patterns and frequently involve non-syntenic as well as syntenic DNA from the same chromosomal region and can harbor multiple genes likely to be associated with tumorigenesis [5–7]. In breast cancer, the 11q13 amplicon can vary in size from less than 1 to 4.5 Mb. This amplicon includes a number of candidate oncogenes including *CCND1* and *EMS1*, which can be amplified independently of each other [2,5]. Similarly, there appears to be two distinct regions on 17q that become amplified in breast cancer; the 17q11 region that harbors *HER-2* and other candidate oncogenes, and the 17q23 region that has been recently described by different laboratories [6,8].

Tumor cell lines are good models for fine mapping of amplified genomic regions and for functional studies of candidate oncogenes because their molecular and cytogenetic aberrations and biological properties reflect distinct subsets of primary tumors. Over the past several years, we have developed a novel panel of HBC cell lines that are ideally suited for elucidating molecular biologic characteristics of breast cancer [9]. In our panel of 11 breast cancer cell lines, we found 3 cell lines, SUM-44, SUM-52, and SUM-225, that have overlapping amplicons centered at 8p11.2. Amplification of the 8p11~p12 region occurs in 10–15% of primary, uncultured HBCs [10], and fibroblast growth factor receptor 1 (*FGFR1*) has long been considered to be the best candidate oncogene at that locus. However, the exact involvement of this receptor in the progression of the cancer is unclear because it is not consistently present in the core-amplified domain and is not always overexpressed when amplified. Identification and characterization of amplified regions can provide important insights into the pathogenesis of breast cancer, and can lead to the identification of targets for novel therapeutics. In this report, we describe in detail the genomic structure of the 8p11~p12 amplicon with molecular cytogenetic analysis. Like other amplicons identified in breast cancers, the 8p11~p12 amplicon has a highly complex

* Corresponding author. Tel.: 734-763-1317; fax: 734-647-9480.
E-mail address: spethier@umich.edu (S.P. Ethier).

structure, and *FGFR1* is not in the common core-amplified domain in 3 breast cancer cell lines with the amplicon.

2. Materials and methods

Our previous chromosome and array comparative genomic hybridization (CGH) studies demonstrated that three of our breast cancer cell lines, SUM-44, SUM-52, and SUM-225 exhibited high-level amplifications at 8p11~p12 [9,11] Furthermore, the SUM-225 cell line contains a separate amplicon at 8q11. The array CGH results and the detailed map of the 8p11~p12 and 8q11 amplicon in SUM-44, 52, and 225 based on the April 2003 freeze of the human genome sequence (UCSC) is shown in Table 1.

In an effort to more carefully define the genomic structure of the amplicons in these three cell lines, and to better understand the mechanistic basis for the copy number increases observed in them, fluorescence in situ hybridization (FISH) analyses were carried out using BAC probes that map to the 8p11~p12 and 8q11 regions. We selected five commercially available BACs from the completed chromosome 8 sequence as probes for the 8p11~p12 region: RP11-701H6 (*ADRB3* locus), RP11-350N15 (*FGFR1* locus), RP11-723D22 (*TACC1* locus), RP11-44K6 (*INDO* locus), and RP11-470M17 (*TC-1* locus) (Fig. 1). One BAC, RP11-217N16, from the 8q11.1 region was also used in the SUM-225 line. A chromosome 8 centromeric probe, CEP8, was used as a control.

Metaphase chromosome slides were prepared from the SUM series of HBC cell lines using standard methods. Chromosomal in situ suppression hybridization and fluorescent detection of hybridization signals were carried out as described previously [12]. The copy number and molecular organization of the region of interest were assessed according to the hybridization patterns observed on both metaphase and interphase chromosomes.

3. Results

In the SUM-44 cell line, all 8p11~p12 BACs yielded clustered FISH signals on 2 marker chromosomes (Fig. 2). In addition, 2 apparently normal copies of chromosome 8 were present in most metaphase spreads. By contrast, only 4 FISH signals on most metaphase and interphase spreads were detected with the centromeric (CEP8) probe. In metaphase spreads, the hybridization pattern of the BAC probes and CEP8 signals were always located on the same marker chromosomes with 8p11~p12 amplification. FISH combined with array CGH analysis revealed the amplified 8p11~p12 region in SUM-44 to be intrachromosomal and to involve several megabases of syntenic sequences (Figs. 1–2). Based on the FISH hybridization pattern and our previously published karyotype of SUM-44 [13], we suggest a simple primary structural model of the 8p11~p12 amplicon in this cell line. During the tumorigenic process, the 8 p11~p12 region of one chromosome 8 underwent in situ amplification resulting

Table 1
Mapping profile of array-CGH within 8p11~12 and 8q11 region in 3 cell lines

BAC Clone	Cytoband	Gene	Base start	Base end	SUM-44	SUM-52	SUM-225
RP11-139G9	8p12	*WRN*	30886709	30928744	−0.72	−0.84	−1.30
CTD-2020E14	8p12		31062838	31063263	−0.73	−0.82	−1.42
RP11-5713	8p12	*NRG1*	32268599	32429032	−0.77	**1.81**	−1.45
RP11-122D17	8p12	*NRG1*	32435232	32609814	−0.83	**1.88**	−1.51
RP11-258M15	8p12		33377743	33538816	−0.73	**2.34**	−1.26
RP11-274F14	8p12	*UNC5D*	35328931	35329267	−0.85	**1.65**	−0.49
RP11-237M13	8p12	*UNC5D*	35433788	35608399	−0.77	**1.11**	−0.61
RP11-210F15	8p12		36250826	36251210	ND	**1.02**	−0.64
RP11-265K5	8p12	*HTPAP, WHSC1L1, FLJ25409, FGFR*	37849071	38026421	ND	**1.28**	0.69
RP11-100B16	8p12	*FLJ25409, FGFR1*	37980628	38144207	ND	**1.34**	0.74
RP11-262I23	8p11.21	*INDO*	39468496	39667003	**1.52**	**1.43**	**2.22**
RP11-133O7	8p11.21		39578684	39626434	**1.89**	**1.58**	**2.32**
RP11-284J3	8p11.21		40384739	40385032	**1.06**	**1.61**	−1.42
RP11-282J24	8p11.21		41386548	41386797	**1.52**	**1.40**	−1.32
RP11-64C22	8p11.21		42090855	42125967	0.08	**1.47**	−1.15
RP11-73M19	8p11.21	*FLJ32731, FLJ22242*	42724620	42892080	0.07	0.72	−1.22
RP11-12L15	8q11.1		47495880	47647566	−0.03	−0.12	**3.36**
RP11-217N16	8q11.21		47715320	47715660	0.08	−0.08	**3.34**
RP11-268N2	8q11.21		48790973	48808994	−0.57	−0.03	−1.07
RP11-149G12	8q11.21		51871742	51872129	−0.39	0.12	−0.78

The BAC clones in array-CGH are ordered by cytoband and base-pair position, and the log2 ration of each BAC probe is given for each cell line. Boldface indicates ratios greater than or equal to 1.00 as genomic amplification. The BAC's position and genes within each BAC clone were obtained from the UCSC database (http://genome.ucsc.edu).

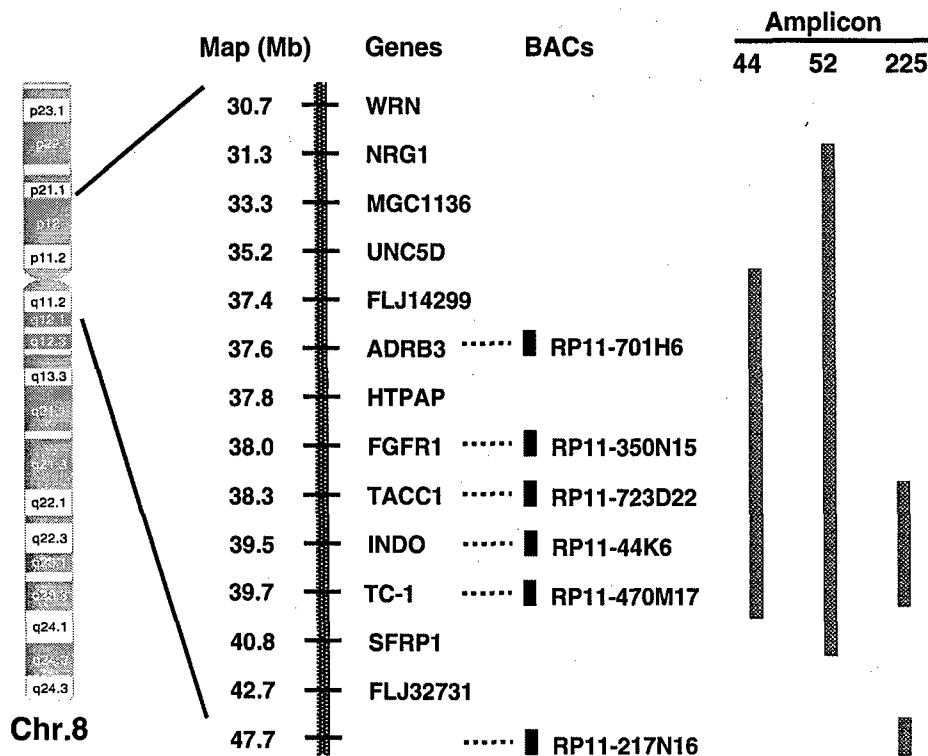*Abbreviation:* ND, not determined.

			Amplicon		
Map (Mb)	Genes	BACs	44	52	225
30.7	WRN				
31.3	NRG1				
33.3	MGC1136				
35.2	UNC5D				
37.4	FLJ14299				
37.6	ADRB3 ······ ▮ RP11-701H6				
37.8	HTPAP				
38.0	FGFR1 ······ ▮ RP11-350N15				
38.3	TACC1 ······ ▮ RP11-723D22				
39.5	INDO ······ ▮ RP11-44K6				
39.7	TC-1 ······ ▮ RP11-470M17				
40.8	SFRP1				
42.7	FLJ32731				
47.7	······ ▮ RP11-217N16				

Chr.8

Fig. 1. Ideogram of 8p11~p12 amplicon in 3 HBC cell lines: SUM-44, SUM-52, and SUM-225. The position of representative genes based on UCSC database (http://genome.ucsc.edu), and BACs used for FISH are presented to the right of the chromosome 8 ideogram. The amplicon in each cell line is based on the results of array-CGH, FISH, and Southern blot analysis.

in a large tandem duplication of the region. Subsequently the chromosome containing the 8p11~p12 amplified chromosome and the normal chromosome 8 were duplicated resulting in two normal chromosome 8s and two chromosome 8s with the amplicon.

FISH patterns from SUM-225 cells resembled those from SUM-44 cells in that copy number increases appear to have resulted from the generation of clustered DNA amplification. However, the regions of focal gene amplification in SUM-225 differ from those of SUM-44 cells. In the SUM-225 cell line, FISH (Fig. 3) and array-CGH (Table 1) analysis revealed two separate amplified regions; one at 8p11~p12 and a second at 8q11.

In metaphase spreads, clustered FISH signals obtained with the 44K6 and 470M17 probes were present on 2–4 marker chromosomes (Fig. 3, top left panel) and more than 18 FISH signals were counted in interphase spreads, while BAC probe 723D22 (*TACC1* locus) yielded 7 to 12 signals in metaphase and interphase chromosome spreads. In contrast, probes 701H6 (Fig. 3, bottom right panel) and 350N15 yielded essentially background signals. Interestingly, BAC probe 217N16, located at 8q11.1, also showed high-level amplification in metaphase and interphase nuclei (Fig. 3, bottom left panel), and clustered FISH signals were also observed in 3–6 marker chromosomes in each metaphase spread.

Two-color FISH with 470M17 and 217N16 revealed the 8q11 and 8p11 probes have coamplified FISH signals in 2–4 marker chromosomes, whereas the 8q11 probe has independent amplified signals in 1–3 other marker chromosomes. The BAC probe 217N16 from 8q11.1 actually overlaps with the centromere region, and therefore, it is not surprising that the centromeric CEP8 probe also showed increased signals on several marker chromosomes (Fig. 3, top right panel). This suggests that SUM-225 cells share a small common region of gene amplification with the SUM-44 and SUM-52 cells in the 8p11 region, and that they also have a separate unique region of gene amplification across the centromere involving the proximal 8q11 region. Because the 8q11 amplicon has the highest level of amplification in SUM-225 cell line, it may also harbor uncharacterized breast cancer genes. As we can only look at the end product of the amplification process, we do not know if the two amplicons within 8p11 and 8q11 regions of SUM-225 were initiated as a large amplified DNA fragment followed by a secondary rearrangement, resulting in two separate amplicons, or if the two domains were co-amplified in some marker chromosomes and independently in others.

The molecular cytogenetic alterations present in the SUM-52 cells are considerably more complex than for the SUM-44 or SUM-225 cell lines. Previous chromosome banding analysis showed SUM-52 had complex karyotypes
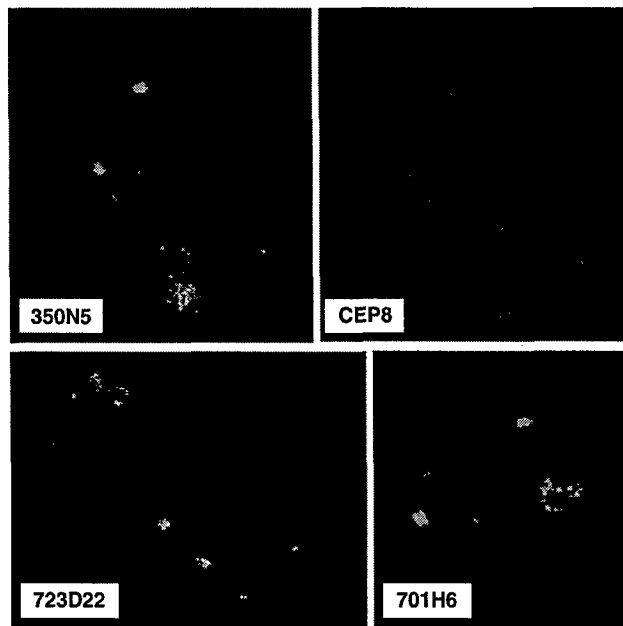
Fig. 2. Representative images of FISH analysis of the 8p11 amplicon demonstrate high-level amplification of the 8p11~p12 region in SUM-44 HBC cells. The BACs of 8p11~p12 region were amplified on 2 marker chromosomes and in more than 20 signals in interphase cells. There are only 4 chromosome 8 centromere signals (red) in the displayed metaphases and interphase spreads.
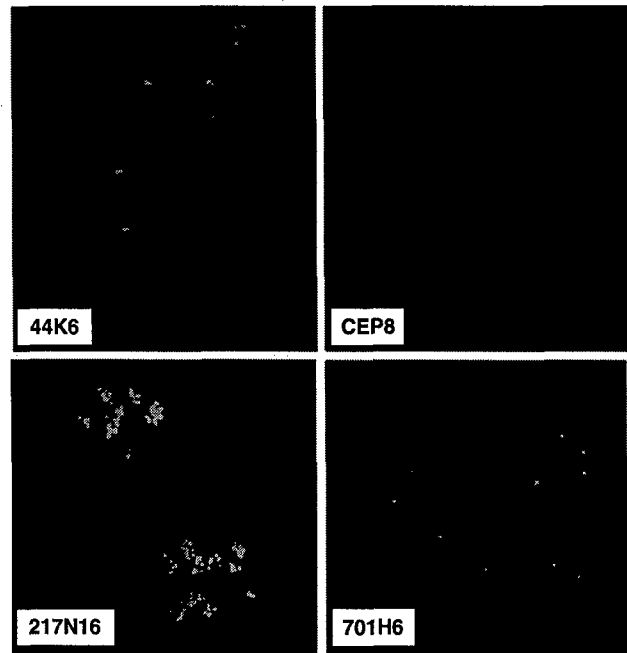

Fig. 3. Representative images of FISH analysis of the 8p11 amplicon demonstrate the two separate peaks of amplification in SUM-225 cells. The 44K6 BAC shows 8p11 amplification, however the 701H6 BAC does not show amplification. The centromeric probe and the 217N16 BAC from 8q11 also show amplification of sequences from the opposite side of the centromere at proximal 8q11.

with multiple numerical and structural aberrations [14]. The representative karyotype has one normal chromosome 8 with many marker chromosomes (http://www.cancer.med.umich.edu/breast_cell/Production/sumlines/karyotypes/Sum-52PE Karyotypes.html). In the FISH hybridization, the signal intensity and hybridization pattern of the five 8p11~p12 BAC probes in SUM-52 interphase and metaphase spreads were complex and heterogeneous. Twelve to 20 copies of the 5 BAC probes were detected in metaphase chromosomes and interphase nuclei, whereas 8 to 12 copies of the CEP8 probe were observed. In most metaphase spreads, intense signals of BAC probes were present on 1–3 marker chromosomes, suggesting clustered duplication of the corresponding genomic segment (Fig. 4c and d).

Interestingly, CEP8 signals were not detected on these marker chromosomes. However, BAC and CEP8 signals were detected in 2–3 cytogenetically normal chromosomes 8 (Fig. 4a). In addition, CEP8 signals were detected in 4–8 chromosomes that did not yield signals with 8p11~p12 BAC probes (Fig. 4b). These CEP8 signals were generally fainter and smaller than the corresponding normal centromeric signals. The fact that several derivative chromosomes showed only 8p11~p12 BAC or CEP8 signals in FISH hybridization, suggests that breaks and translocations in 8p11~p12 likely occurred during the amplification process. Indeed, Adelaide et al. has recently demonstrated that SUM-52 cells have 8p12 amplification and 8p12~ptcr loss with breakpoints in the NRG1 gene [15]. Thus, unlike SUM-44 and SUM-225 cells,

SUM-52 cells have gene amplification as a result of more complex translocation and rearrangement. The presence of nonsyntenic amplified regions of the genome in the SUM-52 and SUM-225 cells suggests that the amplification process resulted from a combination of molecular events.

4. Discussion

Gene amplification is a frequent event in human cancers, but little is known regarding the mechanism of gene amplification or how the overall genomic structure that constitutes the amplified DNA is assembled. From the detailed studies of in vitro model systems of drug-resistant cell lines, it is generally agreed that at least two different mechanisms can drive amplification [16–18]. One is a breakage-fusion-bridge (BFB) cycle mechanism that accumulates copies organized as large repeats on a chromosome arm where one normal gene copy maps in non-amplified cells [16,19]. Second, the amplified DNA can megabase long extra-chromosomal DNA sequences called double minute. The BFB mechanism has proved relevant to the specific breakage of genomic DNA at fragile sites that are points at which chromosomes break non-randomly under certain specific conditions [18–21]. Coquelle et al. have found that fragile sites trigger intrachromosomal gene amplification and form the boundaries of amplicons [16]. In addition, gene amplification mediated by BFB cycles at fragile sites has been demonstrated
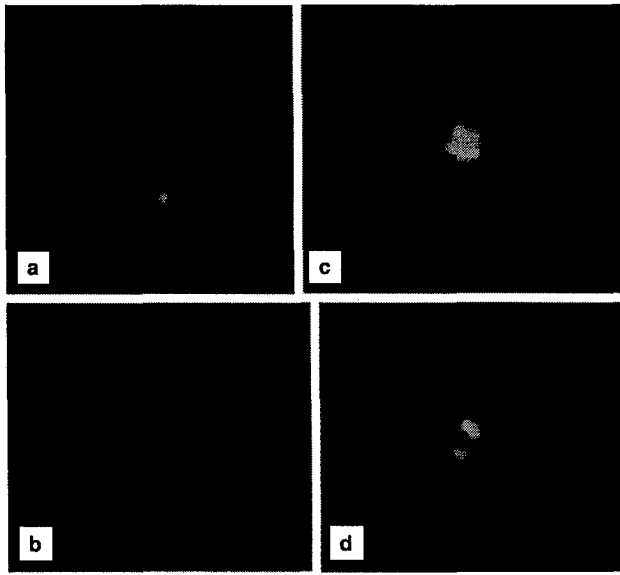
Fig. 4. Representative images of FISH analysis of the 8p11 amplicon demonstrate representative marker chromosomes from SUM-52 metaphase FISH experiments using the 350N15 BAC probe and the centromeric probe (CEP 8). Displayed are: an apparently normal chromosome 8 (a), a marker chromosome with only the CEP8 signal (b), and marker chromosomes with multiple signals from the 350N15 BAC probe (c,d).

in human cancer, such as for the MET oncogene in gastric cancer through FRA7G, and for the RIN gene in oral cancer through FRA11B [22,23].

The 8p11~q11 region has been reported as a common fragile site [24]. This suggests 8p11~p12 and 8q11 amplification in our cell lines appears to have resulted from BFB cycles at the 8p11~q11 fragile site. Our data from FISH, array-CGH, and Southern blot analyses, indicate that extensive DNA rearrangement and loss of intervening DNA may have taken place during the evolution of the 8p11~p12 amplification in SUM-52 and SUM-225 cell lines [11]. Our observations are in line with those made by Adelaide et al., who found the 8p12~p21 region is particularly complex with at least 7 different breakpoint targets within the NRG1 gene in breast and pancreatic cancer cell lines [15]. The genesis of such complex abnormalities cannot be fully explained by BFB cycles and likely involves additional breakage and recombination events at both fragile sites and non-fragile site regions.

The results of the FISH studies were consistent with and extended the information gained from the conventional CGH and array-CGH analyses performed previously, and confirmed that FGFR1 is beyond the core-amplified domain in SUM-225 cell line. Detailed expression profiling of the amplicon in our 3 breast cancer cell lines using a chromosome 8 cDNA microarray and northern blot analysis revealed that FGFR1 is overexpressed at the message level only in the SUM-44 line [11]. Several genes including TACC1, C8ORF4 (TC-1) and other genes within common core-amplified domain have been found to be overexpressed in breast cancer cell lines and primary tumors [11,25].

Our results suggest that the 8p11~p12 region, which has a similar complex amplification pattern as those observed at 20q12, 17q22~q24, and 11q13, may contain more than one important gene. Co-selection and a synergistic role of two or more genes may occur in the development and progression of some breast cancers. Further studies of the amplification and expression of candidate breast cancer oncogenes in a large set of primary breast cancers, as well as determination of their function in cell transformation, will be necessary to address the relationship between these genes and breast cancer progression.

Acknowledgments

References

[1] Deming SL, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br J Cancer 2000;83:1688–95.

[2] Hui R, Campbell DH, Lee CS, McCaul K, Horsfall DJ, Musgrove EA, Daly RJ, Seshadri R, Sutherland RL. EMS1 amplification can occur independently of CCND1 or INT-2 amplification at 11q13 and may identify different phenotypes in primary breast cancer. Oncogene 1997; 15:1617–23.

[3] Borg A, Baldetorp B, Ferno M, Olsson H, Sigurdsson H. c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. Int J Cancer 1992;51:687–91.

[4] Kreipe H, Feist H, Fischer L, Felgner J, Heidorn K, Mettler L, Parwaresch R. Amplification of c-myc but not of c-erbB-2 is associated with high proliferative capacity in breast cancer. Cancer Res 1993;53: 1956–61.

[5] Ormandy CJ, Musgrove EA, Hui R, Daly RJ, Sutherland RL. Cyclin D1, EMS1 and 11q13 amplification in breast cancer. Breast Cancer Res Treat 2003;78:323–35.

[6] Barlund M, Tirkkonen M, Forozan F, Tanner MM, Kallioniemi O, Kallioniemi A. Increased copy number at 17q22-q24 by CGH in breast cancer is due to high-level amplification of two separate regions. Genes Chromosomes Cancer 1997;20:372–6.

[7] Tanner MM, Tirkkonen M, Kallioniemi A, Isola J, Kuukasjarvi T, Collins C, Kowbel D, Guan XY, Trent J, Gray JW, Meltzer P, Kallioniemi OP. Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. Cancer Res 1996;56:3441–5.

[8] Sinclair CS, Rowley M, Naderi A, Couch FJ. The 17q23 amplicon and breast cancer. Breast Cancer Res Treat 2003;78:313–22.

[9] Forozan F, Veldman R, Ammerman CA, Parsa NZ, Kallioniemi A, Kallioniemi OP, Ethier SP. Molecular cytogenetic analysis of 11 new breast cancer cell lines. Br J Cancer 1999;81:1328–34.

[10] Adelaide J, Chaffanet M, Imbert A, Allione F, Geneix J, Popovici C, van Alewijk D, Trapman J, Zeillinger R, Borresen-Dale AL, Lidereau R, Birnbaum D, Pebusque MJ. Chromosome region 8p11-p21: refined mapping and molecular alterations in breast cancer. Genes Chromosomes Cancer 1998;22:186–99.

[11] Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, Ethier SP. Genomic and expression analysis of the 8p11–12 amplicon in human breast cancer cell lines. Cancer Res 2004;64:40–7.

[12] Yang ZQ, Imoto I, Fukuda Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano S. Nakamura Y, Inazawa J. Identification of a novel gene, GASC1, within an amplicon at 9p23–24 frequently detected in esophageal cancer cell lines. Cancer Res 2000;60:4735–9.

[13] Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL. Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res 1993;53:627–35.

[14] Ethier SP, Kokeny KE, Ridings JW, Dilts CA. erbB family receptor expression and growth regulation in a newly isolated human breast cancer cell line. Cancer Res 1996;56:899–907.

[15] Adelaide J, Huang HE, Murati A, Alsop AE, Orsetti B, Mozziconacci MJ, Popovici C, Ginestier C, Letessier A, Basset C, Courtay-Cahen C, Jacquemier J, Theillet C, Birnbaum D, Edwards PA, Chaffanet M. A recurrent chromosome translocation breakpoint in breast and pancreatic cancer cell lines targets the neuregulin/NRG1 gene. Genes Chromosomes Cancer 2003;37:333–45.

[16] Coquelle A, Pipiras E, Toledo F, Buttin G, Debatisse M. Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. Cell 1997;89:215–25.

[17] Savelyeva L, Schwab M. Amplification of oncogenes revisited: from expression profiling to clinical application. Cancer Lett 2001;167: 115–23.

[18] Popescu NC. Genetic alterations in cancer as a result of breakage at fragile sites. Cancer Lett 2003;192:1–17.

[19] Richards RI. Fragile and unstable chromosomes in cancer: causes and consequences. Trends Genet 2001;17:339–45.

[20] Sutherland GR. Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science 1977;197:265–6.

[21] Sutherland GR, Baker E, Richards RI. Fragile sites still breaking. Trends Genet 1998;14:501–6.

[22] Hellman A, Zlotorynski E, Scherer SW, Cheung J, Vincent JB, Smith DI, Trakhtenbrot L, Kerem B. A role for common fragile site induction in amplification of human oncogenes. Cancer Cell 2002;1:89–97.

[23] Shuster MI, Han L, Le Beau MM, Davis E, Sawicki M, Lese CM, Park NH, Colicelli J, Gollin SM. A consistent pattern of RIN1 rearrangements in oral squamous cell carcinoma cell lines supports a breakage-fusion-bridge cycle model for 11q13 amplification. Genes Chromosomes Cancer 2000;28:153–63.

[24] Hecht F, Tajara EH, Lockwood D, Sandberg AA, Hecht BK. New common fragile sites. Cancer Genet Cytogenet 1988;33:1–9.

[25] van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.